Assembly and function of cell surface structures of the thermoacidophilic archaeon Sulfolobus solfataricus

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Chapter 3

The bindosome is a structural component of the *Sulfolobus solfataricus* cell envelope

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Abstract

Sugar binding proteins of the thermoacidophile *Sulfolobus solfataricus* function together with ABC transporters in the uptake of sugars. They are synthesized as, precursors with a class III signal peptide that are normally found in archaeal flagellins and bacterial type IV pilins. The functional expression of sugar binding proteins at the cell surface is dependent on the Bindosome assembly system (Bas) that is homologous to bacterial type IV pilin assembly systems. The Bas system consists of an assembly ATPase, BasE; a membrane anchoring protein, BasF; and three small class III signal peptide containing proteins BasABC. Expression of BasEF in a *S. solfataricus* Δ*basEF* strain restored the uptake of glucose, while an ATPase mutant of BasE was unable to complement. BasEF was detergent-extracted from *S. solfataricus* membranes as a stable protein complex. Solute binding proteins can be extracted from the cell surface as two high molecular mass complexes of 700 and 500 kDa, wherein the largest complex also contains the main S-layer protein SlaA. Electron microscopic analysis of the cell surface of the wild-type and Δ*basEF* strain indicates that the absence of the BasEF complex causes an alteration in cell morphology and the corrugation of the S-layer pattern. These results suggest an intimate interaction between the S-layer and the sugar binding proteins that contribute to cell shape.

Chapter 3

Introduction

Sulfolobus solfataricus is a thermophilic crenarchaeon belonging to the family of Sulfolobales and it optimally grows at 80°C and at a pH value of 3-4. It was originally isolated from sulfur rich volcanic areas in the USA, Italy and Iceland and its metabolism and physiology is adapted to these environments (Zillig *et al.*, 1980). From the different strains from Sulfolobales that have been tested for their ability to grow on various minimal media of sugars such as glucose, arabinose and disaccharides such as cellobiose, maltose and lactose, only *S. solfataricus* was found to grow on minimal sugar media (Grogan, 1989). The natural environment of *S. solfataricus* is relatively poor in substrates, which necessitates efficient mechanisms of substrate transport across the membrane. In recent years, the ABC transporters in *S. solfataricus* have been studied extensively and several sugar binding proteins were identified that bind sugars at subnanomolar concentrations (Albers *et al.*, 1999a, Elferink et al., 2001b).

GlcS is a binding protein that binds glucose and galactose, while AraS binds arabinose, fructose and xylose. In bacteria, binding proteins are directed to the surface of the cell by means of an N-terminal signal peptide that upon translocation across the cytoplasmic membrane is removed by a signal peptidase. However, sequence analysis of GlcS and AraS showed that these proteins bear a type-IV pilin-like signal peptide or class III signal peptide at their N-terminus (Albers *et al.*, 1999b, Albers & Driessen, 2002). Class III signal sequence bearing proteins are normally assembled into an extracellular macromolecular complex such as the pilus or flagellar structures. Class III signal peptides are cleaved by a dedicated type-IV signal peptidase which upon processing removes only the positively charged N-terminus leaving a hydrophobic domain of about 20 amino acids attached at the N-terminus of the mature protein (Albers & Driessen, 2002). By the removal of the positive charges, translocation of the protein across the cytoplasmic membrane is possible whereupon the hydrophobic N-terminus acts as scaffold for the assembly of the protein into a supramolecular structure at the extracellular surface of the membrane. *In vitro* cleavage assays showed that the class III signal peptides of GlcS and AraS are indeed processed by the type IV signal peptidase of *S. solfataricus* PibD (Albers *et al.*, 2003). Importantly, the presence of the class III signal peptides predicts that an assembly system is required for the functional expression of the sugar binding proteins at the cell surface. *S. solfataricus* contains five operons that encode subunits that are homologous to components of bacterial Type IV pili assembly systems. Recently, we have shown that the Bas system of *S. solfataricus* is responsible for the functional expression of sugar binding proteins in the cell envelope (Zolghadr *et al.*, 2007). The Bas system consists of the assembly ATPase, BasE; a membrane protein, BasF; and three small proteins BasABC that are synthesized with a class III signal peptide and that possibly form a pseudopilus-like structure. Deletion of the *basEF* and *basABC* genes results in a growth deficiency on various sugars that for uptake depend on a class III signal peptide bearing sugar binding protein. Although the uptake of glucose was impaired in the Δ*basEF* and Δ*basABC* mutants, the translocation of the binding proteins across the cytoplasmic membrane seemed

unaffected suggesting that the Bas system may be involved in correct localization of the binding proteins.

BasE belongs to the family of Type IV pili assembly/type II secretion ATPases (Planet *et al.*, 2001, Peabody *et al.*, 2003, Albers & Driessen, 2005) that share several conserved motifs in the Walker A and B sites, and the Asp and His Box boxes. In the bacterial ATPases, these conserved motifs are located at the C-terminal domain (CTD) that constitutes the ATP binding and hydrolysis site (Possot & Pugsley, 1994, Sandkvist, 2001). PulE is the ATPase component of type II secretion system involved in pullulanase secretion by *Klebsiella oxytoca.* Site directed mutants of the Walker A domain are deficient in pullanase secretion (Possot & Pugsley, 1994). A similar study with PilT of type IV pili involved in retraction motility of *Legionella pneumophila* and *Aquifex aeolicus* resulted in loss of the ATPase activity and a block in pilus retraction (Herdendorf *et al.*, 2002, Sexton *et al.*, 2004). BasF is a membrane protein, that due its similarity with the EspN protein of the type II secretion system of *Vibrio cholerae* (Abendroth *et al.*, 2005), is likely to interact with BasE to constitute the functional assembly system.

Currently, the precise role of the Bas system in the localization of the sugar binding proteins remains unclear. We now show that the uptake of glucose is directly dependent on the ATPase activity of BasE, and that BasE and BasF are interacting proteins. Localisation studies indicate that the sugar binding proteins are contained in high molecular mass complexes that are associated with the S-layer. The deletion of the *basEF* genes does not interfere with complex formation but results in cells with an altered morphology and a distorted S-layer. These data indicate that sugar binding proteins are functionally associated with the S-layer.

Results

Characterization of BasEF by site directed mutagenesis

To determine the role of the ATPase activity of BasE, the conserved lysine residue (K237) of the Walker A motif was replaced by an alanine residue by site directed mutagenesis. In general, the introduction of this mutation in homologous assembly ATPases results in a loss of the ATPase activity and a functional defect (Herdendorf et al., 2002, Sexton et al., 2004). The mutated *basE* gene was subcloned with *basF* into the virus expression vector pSVA of *S. solfataricus* to yield pSVA107. The pSVA107 plasmid was introduced into the Δ*basEF* strain and glucose uptake was measured and compared to a strain transformed with pSVA75 containing the native *basEF* genes (Zolghadr et al., 2007, Albers et al., 1999a). The cells complemented with BasE(K237A)F showed a glucose transport activity similar to the negative control, whereas cells transformed with the wild-type BasEF showed high levels of glucose uptake (Figure 1). The results demonstrate that the ATPase activity of BasE is essential for active glucose transport in *S. solfataricus*.

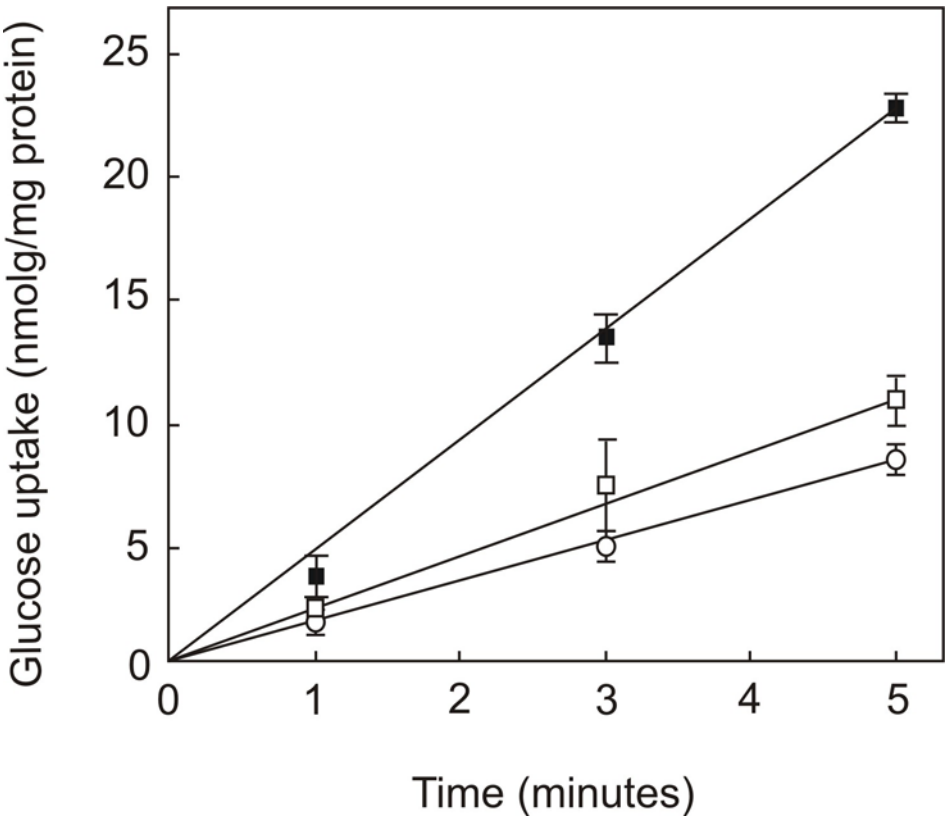

Figure 1. Uptake of glucose by the Δ*basEF* strain without (open circles) and with a plasmid containing BasEF (pSVA75) (closed squares) or BasE(K237A)F (pSVA107) (open squares).

BasE and BasF co-purified as a complex

Since the expression of BasEF in the Δ*basEF* strain restored the glucose uptake, we performed a detergent extraction and purification of the Bas complex from these cells in order to determine the complex subunit composition. Herein, BasE and BasF were co-expressed in *S. solfataricus* using pSVA75 (Zolghadr et al., 2007) with a C-terminal tandem tag (STREP and a 10x his-tag) on BasF. Membranes from the expression strain were isolated and solubilised in 1% Triton-X100 or 2 % dodecyl maltoside (DDM). Solubilized membrane proteins were collected by centrifugation and applied to a nickel NTA affinity column. The bound proteins were eluted with imidazole yielding an elution fraction that contained BasF with the expected molecular size of 75 kDa (Figure 2A) and various other proteins. The identity of BasF was confirmed by immunobloting using an antibody directed against BasF (Figure 2B). The elution fraction contained another protein with a size of ~ 60 kDa (Figure 2A), which was identified as BasE by immuno-blotting using a polyclonal antibody raised against the purified BasE. These data indicate that BasF and BasE are interacting proteins forming a stable membrane protein complex.

To determine if BasEF is present in a high molecular mass complex, the partial purified BasEF complex was subjected to size exclusion chromatography using a Superdex 200 column and 0.1 % dodecylmaltoside (Figure 3A). Fractions were analyzed on SDS PAGE (Figure 3B), and by immuno-blotting with BasF and BasE antibodies (Figure 3C and D, respectively). The first elution peak with an approximate mass of 1 MDa contained a 60 kDa protein of unknown identity (Figure 3B, lane 1).

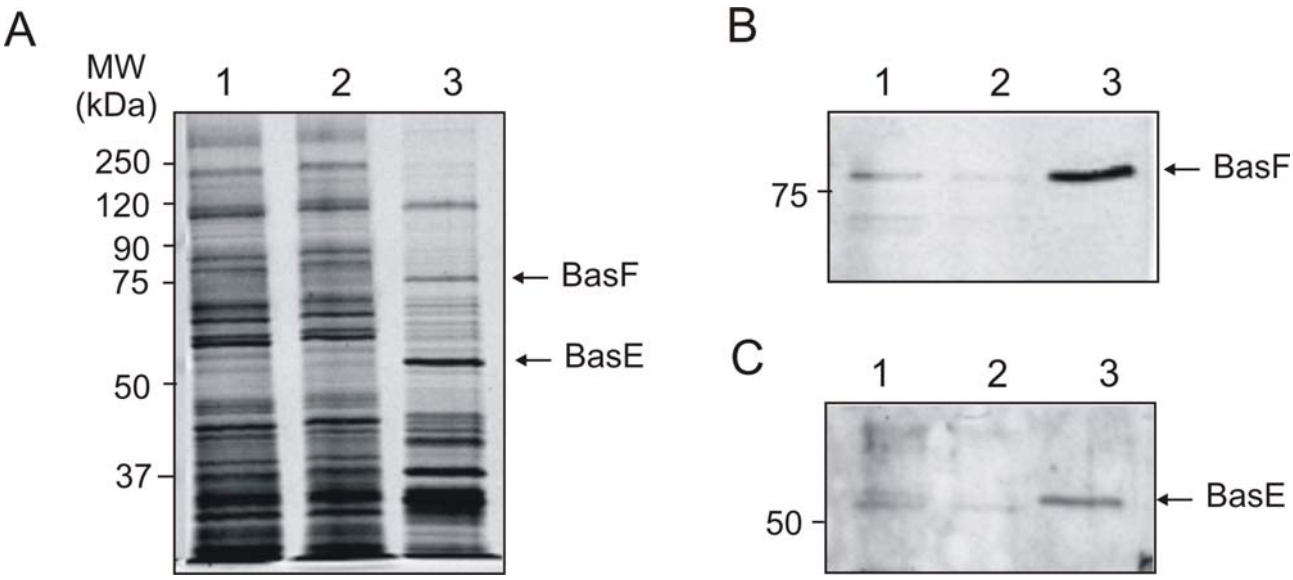

Figure 2. Co-purification of the BasE and BasF proteins expressed in *S. solfataricus* Δ*basEF* cells. Isolated membranes were solubilised in 2% DDM, and solubilised proteins were passed over a Ni-NTA affinity column and eluted with .. mM imidazole. Eluted fractions were analyzed on SDS-PAGE (A), and western blotting using antibodies against BasF (B) and BasE (C).

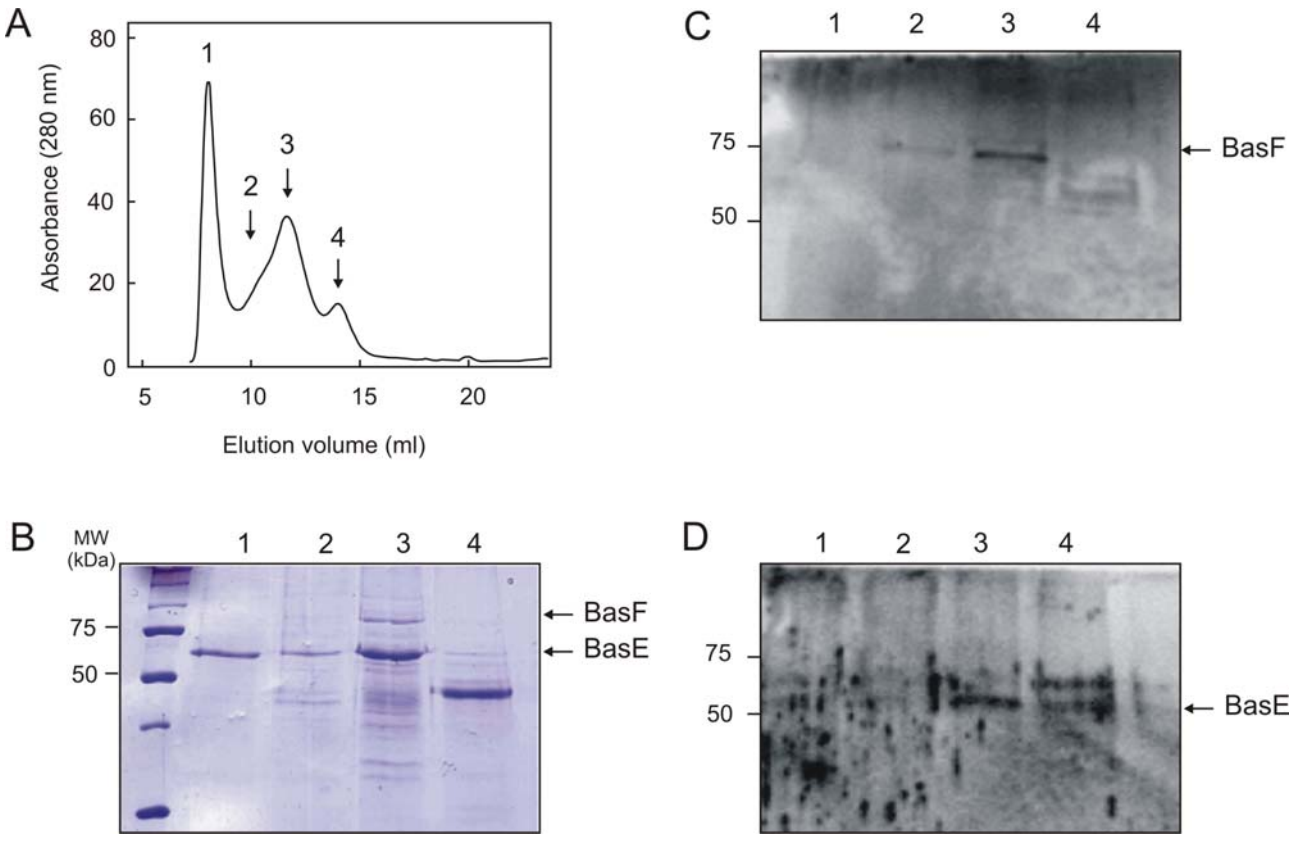

Figure 3. Size-exclusion analysis of the elution fraction from the Ni-NTA affinity purification of the BasEF complex (A). Collected fractions were analyzed on SDS-PAGE (B) and western blotting using antibodies against BasF (C) and BasE (D).

The second peak, which corresponded to a size of 100 – 200 kDa, contained the BasE and BasF proteins (Figure 3B, lane 3). The third peak contained a protein with a mass of approximate 40 kDa (Figure 3B, lane 4). Mass spectroscopy analysis failed to reveal the identity of the 60 and 40 kDa proteins. The estimated size of the BasEF elution fraction was smaller than expected. A stoichiometric complex of BasEF is predicted to have a molecular mass of ~ 135 kDa. However, ATPases homologous to BasE form a hexameric ring (Herdendorf et al., 2002, Yamagata & Tainer, 2007), and thus a putative BasE hexamer alone would correspond to a mass of ~ 360 kDa. The amount of BasF molecules in the complex is unknown, but considering that the BasF is 75 kDa while taking the detergent micelle into account, a minimum size of 500 kDa is expected for the BasEF complex. Therefore, the eluted fraction mostly likely corresponds to a stoichiometric BasEF complex.

Sugar binding proteins on *S. solfataricus* are assembled into high molecular complexes

To determine if the sugar binding proteins are contained in large complexes at the cell surface, binding proteins were extracted from membranes derived from *S. solfataricus* wild type and Δ*basEF* cells using a low Triton X-100 concentration, and partially purified by ConA lectin affinity chromatography. When analyzed by Blue native PAGE, the ConA enriched binding proteins were found in two main complexes with apparent molecular masses of about 600 and 400 kDa, respectively (Figure 4). In addition several smaller complexes were observed. The binding proteins isolated from the Δ*basEF* strain showed a similar complex formation, albeit their levels were reduced relative to the wild-type strain. The complexes observed in the BN-PAGE showed a distinct protein composition on SDS-PAGE. Protein complex 1 contained the main S-layer protein (SlaA) and a protein at 120 kDa that by mass spectrometry was identified as a putative dipeptide binding protein, SSO1273 (Figure 4C). SlaA has previously been detected to represent a major component of the ConA purification fraction (Ellen *et al.*, 2009) and found to be glycosylated (data not shown). Protein complex 2 consists of the glucose binding protein (GlcS) and another putative dipeptide binding protein SSO3043 (Figure 4C). Both, SSO3043 and SSO1273, are synthesized with a type I signal peptide. It should be emphasized that the annotation of SSO304 and SSO1273 as putative dipeptide binding proteins may not necessarily mean that these proteins bind dipeptides as previous analysis of such proteins revealed that most represent sugar binding proteins (Elferink et al., 2001b, Nanavati *et al.*, 2006).

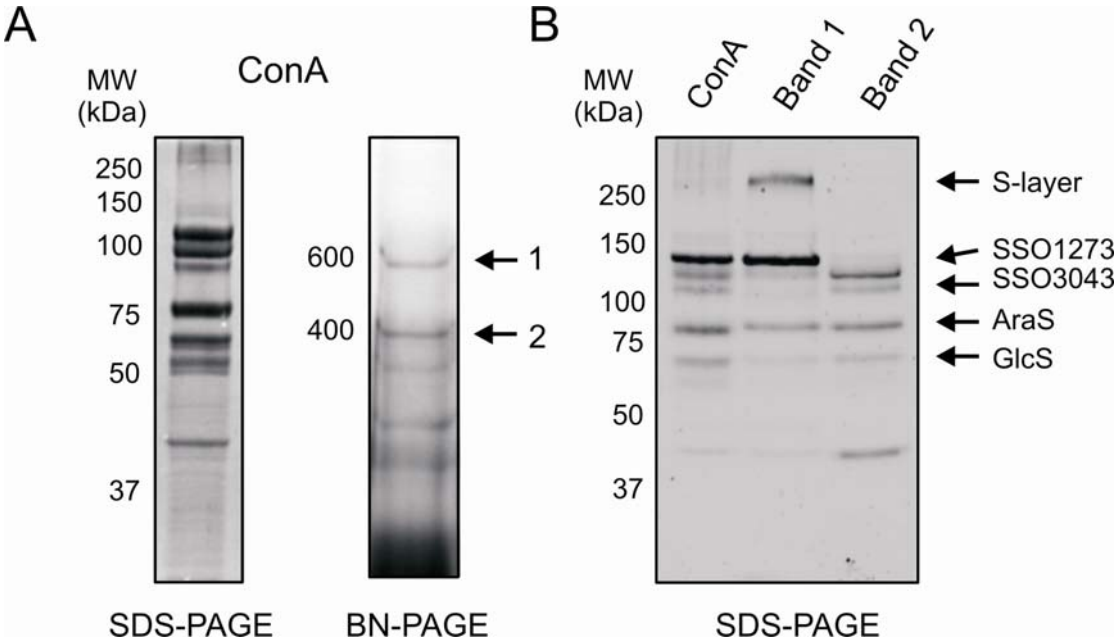

Figure 4. Blue-native PAGE analysis of the sugar binding proteins extracted from membranes derived from *S. solfataricus*and purified with ConA lectin affinity purification (A). The sugar binding proteins are present in two complexes with molecular masses of 400 and 600 kDa, respectively. The bands from Blue-native gel were analysed by SDS-PAGE (B) and protein bands were identified by mass spectrometry.

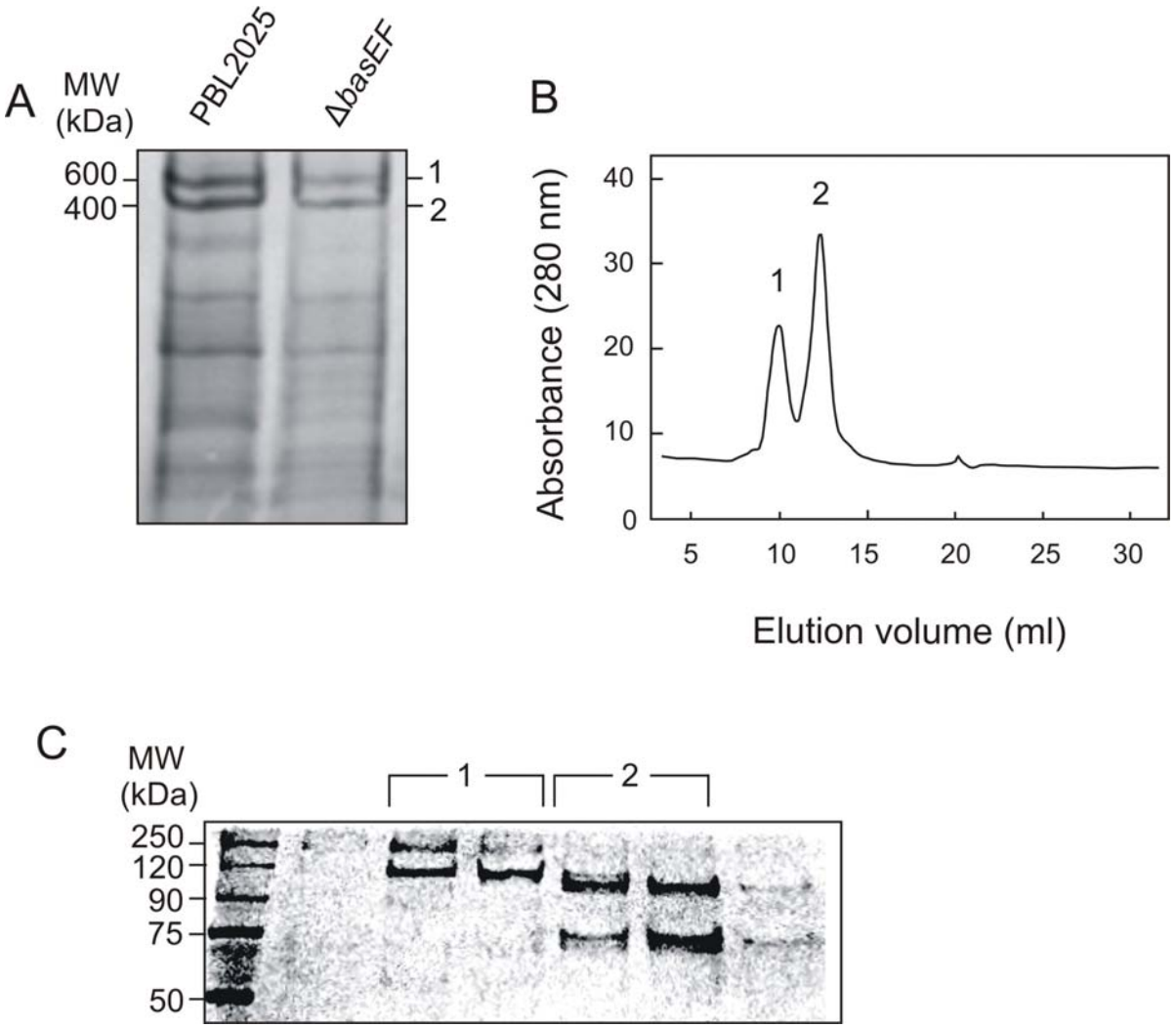

Figure 5. Blue-natibe gel analysis (A) and Size-exclusion chromatographic analysis (B) of the sugar binding proteins extracted from membranes derived from *S. solfataricus* wild-type and Δ*basEF* cells and purified with ConA lectin affinity purification. The peak fractions were analysed by SDS-PAGE (C) and protein bands were identified by mass spectrometry.

Especially, SSO3043 is surrounded by enzymes annotated to be involved in sugar degradation. Interestingly, when the growth medium was supplemented with 0.4% arabinose, the arabinose binding protein (AraS) was induced and found to be part of protein complex 2 on BN-PAGE (Figure 4A and C, Band 2). These results demonstrated that the sugar binding proteins isolated from the cell surface of *S. solfataricus* are contained in high molecular mass complexes, although complex formation seems to occur independently of the Bas system. The ConA elution fractions were also analysed by size exclusion chromatography (Figure 5) to estimate the native mass of these complexes. The proteins eluted in two major peaks with apparent molecular masses of 700 and 500 kDa, respectively. The protein complexes were collected and analyzed on SDS PAGE and stained with syber ruby, a sensitive fluorescent staining method (Figure 5B). The analysis confirm the observations made by BN-PAGE showing that the 700 kDa complex

corresponds to complex 1 with SlaA and SSO1273 as main subunits. The 500 kDa protein complex contained GlcS and SSO3043 (Figure 5B) and corresponds to complex 2. These data demonstrate that the (sugar) binding proteins are contained in high molecular mass complexes with a relatively uniform size. The presence of SlaA in one of these complexes suggests that the binding proteins are associated with the S-layer.

Analysis of *S. solfataricus* surface layer

In an attempt to visualize the bindosome structure in the cell envelope of *S. solfataricus*, we examined the cell surface by transmission electron microscopy (TEM). Herein, cells were subjected to high pressure freezing, freeze substitution and TEM to visualize highly defined structures of the cell envelope of the PBL2025 control strain and the Δ*basEF* mutant strain. TEM images showed a well-preserved cell surface from both strains in ultra-thin sections (Figure 6). Interestingly, the morphology of wild-type cells appeared as highly lobed and irregular shaped cocci while the Δ*basEF* cells were less lobed, almost regular cocci (Figure 6A and B). Possibly, these structural changes in the cell envelope resulted from the *basEF* deletion. The membrane and S-layer were clearly distinguished and enclose a quasi-periplasmic space with 25 nm in width (Figure 6C and D). The periplasmic space appeared well contrasted, due to the exposure of the cells to OsO_4 and uranyl acetate during the freeze-substitution. This indicates the presence of an abundance of biologically relevant components. Immuno-gold labelling was performed to study the localization of binding protein in the cell envelope. However, the antibodies used to localize GlcS, the glucose binding protein, gave very poor labelling, while another antibody against the A_1A_O ATPase (Küper et al. 2010) resulted in significant and dense labelling of the cytoplasmic membrane (data not shown). Therefore, no final conclusion about the localization of GlcS could be obtained.

In another approach, the surface layer of the PBL2025 and Δ*basEF* cells was visualized by freeze-etching freshly grown cells (Figure 7). In these freeze-etch preparations, the S-layer of strain PBL2025 showed a crystalline lattice of pseudo-hexagonal symmetry, reflecting S-layer protein complexes with internal three-fold symmetry, arranged on hexagonal lattice, in a similar manner as described (Figure 7A and C). The crystalline lattice of the Δ*basEF* S-layer appeared to be less corrugated, (Figure 7B and D). These data can be taken as an indication that the deletion of *basEF* results in a disturbance of the surface layer lattice, and this may have led to different cell morphologies and possibly an altered localization of the sugar binding protein complexes.

Discussion

Here, we have explored several approaches to study the function of the Bas system and its role in the correct localization of the sugar binding proteins in the cell envelope of *S. solfataricus*. The Bas system plays an important role in the functional assembly of sugar

transport in *S. solfataricus*. This is evident from the sugar transport defect of the *basEF* deletion mutant that can be complemented by the expression of BasEF. However, expression of BasE(K237A)F with a mutation of the catalytic lysine residue of the ATP binding site of BasE did not result in complementation. This implies that the ATPase activity of BasE is required for the correct localization and/or assembly of the sugar binding proteins. In a previous study we could demonstrate that BasEF are not needed for the translocation of sugar binding proteins across the cytoplasmic membrane (Zolghadr et al., 2007). Moreover, in the absence of BasEF, the extracellular binding proteins still bind sugars with high affinity. However, these cells are unable to grow on sugars that for uptake depend on a binding protein that is synthesized with a class III signal peptide, and also sugar transport is impaired.

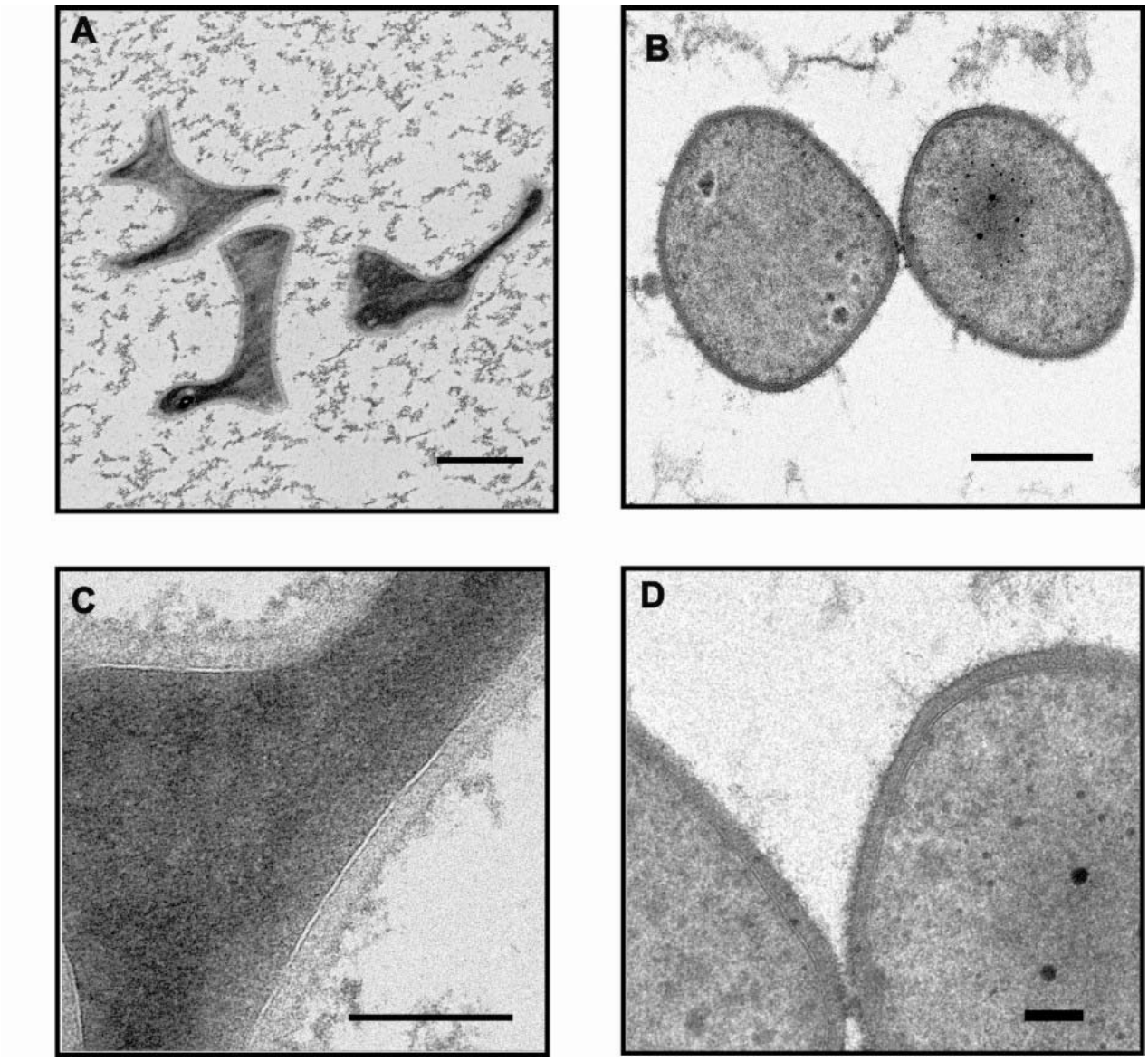

Figure 6. Transmission electronmicroscopy of ultra-thin sections of PBL2025 (A and C) and Δ*basEF* (B and D) cells prepared by high pressure freezing, freeze substitution and embedding in Epon. Sizes of the bars in A and B are 500 nm, C is 200 nm and C is 100 nm.

BasE and BasF are homologous to subunits of bacterial type II secretion and type IV pili assembly systems. BasE is a cytosolic ATPase and BasF is a membrane protein that likely functions to anchor BasE to the membrane. Indeed, BasE and BasF form a complex, but this complex seems labile under the detergent extraction conditions tested, and size exclusion chromatography hints at the presence of a stoichiometrical complex rather than a hexameric arrangement of the BasE subunit. Stabilization of the BasEF complex for future structural analysis remains challenging as the complex is readily degraded by endogenous proteases (unpublished results). An analysis of the ConA purified sugar binding proteins by BN-PAGE and size exclusion chromatography revealed that these proteins are present in a large complex. These complexes could be separated as symmetric peaks on size exclusion chromatography. However, the binding proteins purified from the Δ*basEF* strains were present in a similar complex indicating that BasEF is not required for complex formation per se.

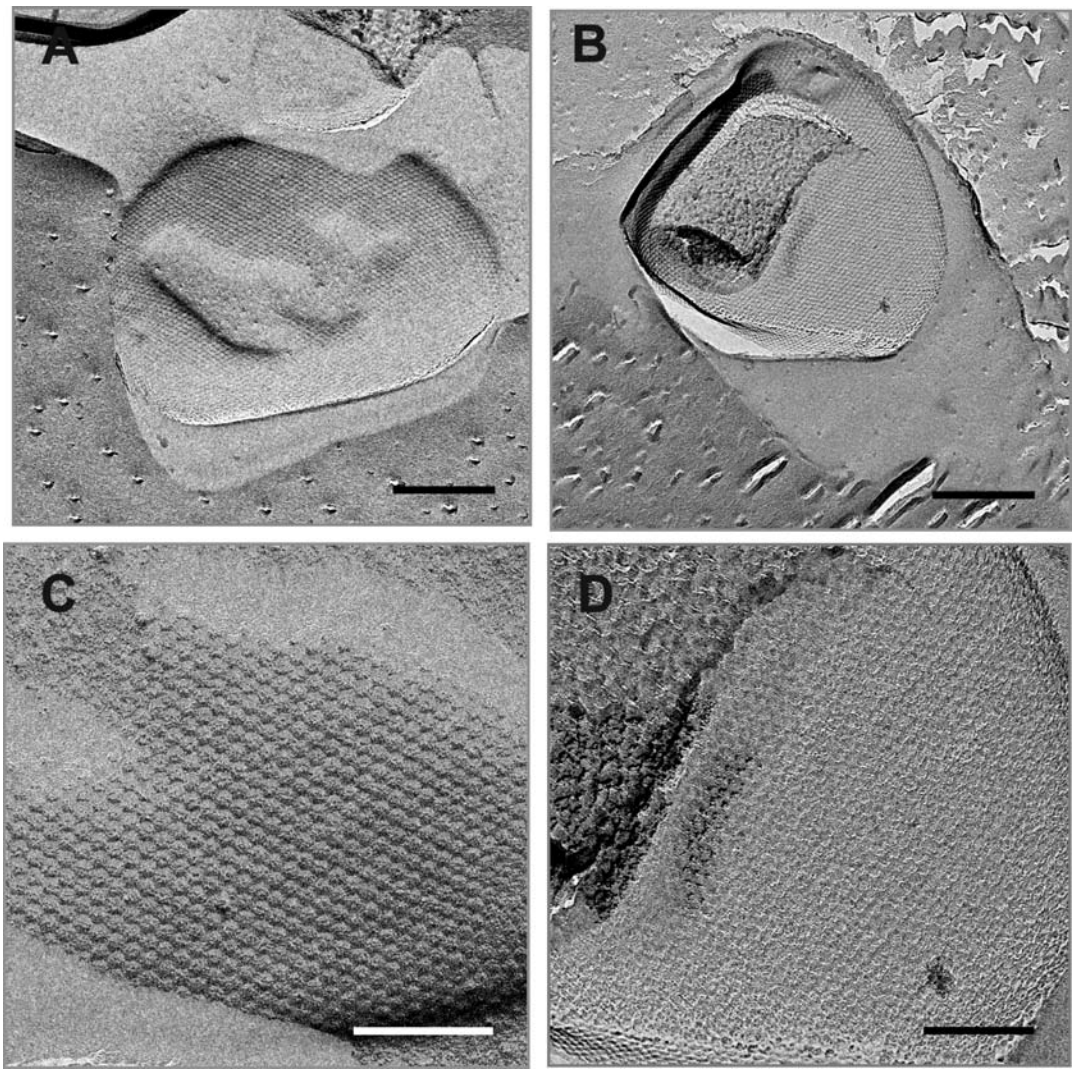

Figure 7. Electron micrographs of freeze-etched S-layers of the BPL2025 (A and C) and Δ*basEF* (B and D) strains. Sizes of the bars are in A and B 500 nm and C and D 200 nm.

Possibly the role of BasEF is to correctly localize the binding protein complexes at the surface layer envelope since part of the binding proteins was complexed with the major S-layer protein SlaA. Analysis of the surface envelope by high pressure freezing yielded high-resolution images of cytoplasmic membrane, quasi-periplasmic space and S-layer. The TEM images of the S-layer suggested that the Bas system does not just generate a "traditional" filament comparable to flagella or pili, but that it rather is involved in or contributes to shaping the cell envelope. In this respect, the sugar binding complexes isolated from the cell surface may represent partially dissociated complexes and part of a large native structure in the cell envelope that is a structurally associated with the S-layer. The quasi-periplasmic space of *S. solfataricus* is composed of dense, biologically relevant macromolecules, most probably glycan chains from lipids, glycosylated proteins and extracellular proteins (see, e.g. the scheme of the S-layer architecture of *Halobacterium spp*. (Kessel *et al.*, 1988)), which may be essential for the overall stability of cell morphology and cell wall. Such high density may limit the diffusion of extracellular solutes, and assuming that this space amongst others is filled with sugar binding proteins, a specific organization of these binding proteins may facilitate the diffusion of solutes from the outer surface of the S-layer to the cytoplasmic membrane as it is known from Gram-negative bacteria (Brass et al., 1986).

Experimental methods

***S. solfataricus* growth conditions.** *S. solfataricus* PBL2025 (Schelert *et al.*, 2004) and the Δ*basEF* strain were grown in Brock medium at pH 3 and 80°C (Brock *et al.*, 1972) and the medium was supplemented with 0.1(w/v) % of trypton as sole carbon and energy source. Growth of cells was monitored by measuring the optical density at 600 nm.

Construction of *basE* mutant. The lysine residue 237 in the Walker A site of BasE was replaced by an alanine by site-directed mutagenesis. The pMZ11 plasmid harboring the *basEF* genes (pSVA69) was used as a template for the generation of the mutation using the primers 5'-ACGACATTTAATATAAGAGCATTCCC
TGAA-'3 and 5'-TTTTCAGGGAATGCTCTTATATTAAATGTC-3'. The mutated *basEF* was transferred to the SSV1 virus vector with *BlnI* and *EagI* restriction site as described, yielding the plasmid pSVA107 (Zolghadr et al., 2007).

Isolation of membranes. Cells were harvested at OD_{600} of ~1 and resuspended in 50 mM Hepes buffer at pH 8 and supplemented with 1 mM PMSF and a small amount of DNase I. Cells were broken by 10 cycles of 10 sec sonication and 50 second rest (Soniprep 150, LA Abcoude). Unbroken cells were removed by low spin centrifugation at 4300 rpm and 4°C for 15 minutes, and cytoplasmic membranes were harvested by ultracentrifugation at 100.000x g and 4°C for 1 hour. Membrane pellets were resuspended in 50 mM Hepes buffer at pH 8 and frozen in liquid nitrogen and stored at -80°C.

Purification of binding proteins by ConA chromatography. Isolated membranes from *S. solfataricus* PBL2025 and Δ*basEF* cells were solubilised in buffer A (20 mM Hepes buffer

at pH 7.5, 100 mM NaCl) supplemented with 2 % DDM for 1 hour at room temperature. The solubilised fraction was separated from non-solubilised membranes by ultracentrifugation at 100.000x g and 4°C for 1 hour. Solubilised membrane proteins were passed onto a 1 ml ConA sepharose column equilibrated with 10 ml buffer A. The column was washed with 30 ml buffer A, and bound membrane proteins were eluted with buffer A supplemented with 0.05 % DDM and 150 mM methyl-α-D-mannopyranoside. The flow through, wash steps and elution fractions were analyzed on 12 % SDS-PAGE gel. The uptake of D-[^{14}C]-glucose (250 Ci/mmol) by *S. solfataricus* cells was measured as discussed previously (Albers et al., 1999a).

Fixation, freeze-substitution and embedding of *S. solfataricus* cells. High-pressure freezing of samples was carried out with high-pressure freezer (Leica EM-PACT 2) as described previously (Rachel et al., 2002). Freeze-substitution was performed in acetone, with 2% (w/v) OsO_4 added as a fixative. The samples were freeze substituted in a Leica EM AFS (automatic freeze substitution system, Leica, Vienna, Austria) with pure acetone containing 2% (*w*/*v*) osmium tetroxide and 0.1% (*w*/*v*) uranyl acetate at −90°C for 72 h. Samples were washed for 1 h in acetone at room temperature and infiltrated in Epon 812 (Ted Pella, Inc., USA) for 30 h. and the Epon was polymerized at 60 °C for 24 h. Ultrathin sections were cut with a Leica UCT ultramicrotome and mounted on carbon-coated copper grids and the images were recorded with transmission electron micrographs were taken on a Philips CM 12 operated at 120 kV, equipped with a Gatan TV 673 wide-angle camera (Gatan, München).

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